FEBS Letters 582 (2008) 4117–4124

A novel association of mGluR1a with the PDZ scaffold protein CAL modulates receptor activity

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Received 26 October 2008; accepted 28 October 2008

Available online 21 November 2008

Edited by Gianni Cesareni

Abstract Metabotropic glutamate receptor subtype 1a (mGluR1a) associates with the proteins mediating its receptor activity, suggesting a complex-controlled function of mGluR1a. Here, using glutathione-*S*-transferase pull-down, co-immnoprecipitation and immnoflurescence assays in vitro and in vivo, we have found CFTR-associated ligand (CAL) to be a novel binding partner of mGluR1a, through its PSD95/discslarge/ZO1homology domain. Deletion of mGluR1a-carboxyl terminus (CT) or mutation of Leu to Ala in the CT of mGluR1a reduces the association, indicating the essential binding region of mGluR1a for CAL. Functionally, the interaction of mGluR1a with CAL was shown to inhibit mGluR1a-mediated ERK1/2 activation, without an apparent effect, via the C-terminal-truncated receptor. These findings might provide a novel mechanism for the regulation of mGluR1a-mediated signaling through the interaction with CAL.

Structured summary:
MINT-6797987, MINT-6798009:
NHERF-2 (uniprotkb:Q15599) *binds* (MI:0407) to *mGluR1a* (uniprotkb:Q9R0W0) by *protein array* (MI:0089)
MINT-6798026, MINT-6798048, MINT-6798066:
mGluR1a (uniprotkb:Q9R0W0) *physically interacts* (MI:0218) with *CAL* (uniprotkb:Q9HD26) by *pull down* (MI:0096)
MINT-6797953, MINT-6797970:
NHERF-1 (uniprotkb:O14745) *binds* (MI:0407) to *mGluR1a* (uniprotkb:Q9R0W0) by *protein array* (MI:0089)
MINT-6797935:
CAL (uniprotkb:Q9HD26) *binds* (MI:0407) to *mGluR1a* (uniprotkb:Q9R0W0) by *protein array* (MI:0089)
MINT-6798084:
CAL (uniprotkb:Q9HD26) *binds* (MI:0407) to *mGluR1a* (uniprotkb:Q9R0W0) by *filter binding* (MI:0049)
MINT-6798134:
mGluR1a (uniprotkb:Q9R0W0) *physically interacts* (MI:0218) with *CAL* (uniprotkb:Q9HD26) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-6798158:
CAL (uniprotkb:B4F775) *physically interacts* (MI:0218) with *mGluR1a* (uniprotkb:Q9R0W0) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6798233:
CAL (uniprotkb:Q9HD26) *colocalizes* (MI:0403) with *mGluR1a* (uniprotkb:Q9R0W0) by *fluorescence microscopy* (MI:0416)

Keywords: mGluR1a; CAL; PDZ; Cell signaling; Protein–protein interaction

1. Introduction

mGlu receptors are classified into three groups according to their sequence identity, signal-transduction pathways and pharmacology: group I receptors (mGluR1 and mGluR5), group II receptors (mGluR2 and mGluR3), and group III receptors (mGluR4, mGluR6, mGluR7 and mGluR8) [1]. Currently, six splice variants of mGluR1 are known, termed metabotropic glutamate receptor subtype 1a (mGluR1a)-f according to the different shearing modes of their C termini [2–7]. Analysis of the sequence of mGluR1a has indicated a different structure, a large intracellular C-terminal domain, including a PSD95/discslarge/ZO1homology (PDZ)-binding motif (SSTL). This distinct structure of mGluR1a suggests that the physiological signaling pathway of mGluR1a might be modulated by interactions with PDZ domain-containing proteins. Group I receptors (mGluRI) have been found to associate with the PDZ proteins such as tamalin and Shank3 to regulate receptor intracellular trafficking [8,9]. However, the mGluRI targeting proteins still need to be clarified to further reveal the mechanisms of the various receptor functions. For example, the cellular distribution of tamalin only partially overlaps that of mGluRI [10], so it is possible that some other PDZ proteins are also involved in the regulation of mGluRI activity. To identify proteins that interact with the PDZ-binding motif (mGluR1a-carboxyl terminus (CT)) of mGluR1a, we screened a proteomic array of 96 distinct PDZ-domains derived from a variety of cytosolic proteins and identified

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Abbreviations: PDZ, PSD95/discslarge/ZO1homology; mGluR1a, metabotropic glutamate receptor subtype 1a; GST, glutathione-*S*-transferase; CT, carboxyl terminus; ΔCT, deleted carboxyl terminus; His, hexahistidinetagged; HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WT, wild-type; HA, hemagglutinin; CAL, CFTR-associated ligand

0014-5793/$34.00 © 2008 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2008.10.054

CFTR-associated ligand (CAL) also known as PIST, GOPC, and FIG [11–13], as a novel binding partner of mGluR1a. We further observed that the interaction with CAL inhibits mGluR1a-mediated ERK activation, revealing the functional importance of mGluR1a and CAL association. Our study contributes to the literature on the complexity of mGluR-interacting proteins, suggesting a molecular mechanism of the differences between mGluR1a and its variants.

2. Materials and methods

2.1. Preparation of plasmids and fusion proteins

Rat Flag-mGluR1a, mGluR1a-△CT and human hemagglutinin (HA)-CAL plasmids were kindly provided by Randy Hall (Emory University, GA, USA). CAL-PDZ, wild-type (WT) mGluR1a-CT (the last 25 amino acids of mGluR1a) and mutant mGluR1a-CT-L/A were amplified by PCR using the corresponding oligonucleotides (for mGluR1a-CT-L/A, forward: 5′-AGACACGAATTCGTCCT CTGCACCCCTCCAAATGTA-3′; reverse: 5′-AGACACCTCGAG CTACGCGGTGGAAGAGCTTTGCTT-3′) and inserted into pET-30A and pGEX-4T-1 digested with EcoR1 and XhoI, respectively. The methods for expression fusion proteins were used as previously described [14].

2.2. Cell culture and transfection

All tissue culture medium and related reagents were purchased from Hyclone (UT, USA). BHK and COS-7 cells were maintained in complete medium (Dulbecco's modified eagle medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C/5% CO_2 incubator. To express proteins, cells were split into 10-cm plates, grown to 80–90% confluency, then transfected with 1 μg total DNA mixed with Hifectin II (2 μl) (Applygen Technologies, Beijing, China) and added to 9 ml of serum-free medium followed by 10% FBS after incubation for 4 h. Cells were harvested afer 48 h. Agonist stimulation (100 μM, 30 min) was performed at 37 °C in DMEM before the cells were prepared for co-immunoprecipation and immnofluoresence experiments.

2.3. PDZ array overlay assay

Gridded nylon membranes were spotted with hexahistidinetagged (His)-PDZ-domain fusion proteins (1 μg per bin), as described elsewhere [14]. The membranes were dried, blocked and overlaid with 100 nM glutathione-*S*-transferase (GST) fusion proteins in blot buffer, and the arrays were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-GST antibody (1:3000; Amersham Biosciences, NJ, USA). Interactions of the GST fusion proteins with the various PDZ domains were visualized via chemiluminescence using an ECL kit.

2.4. Co-Immunoprecipitation and Western blotting

Cells were harvested and lysed in 1 ml of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.5% Triton X-100, pH 7.4). Lysates were solubilized, clarified and then incubated with an anti-Flag affinity gel (Sigma–Aldrich, UT, USA). After washing with an ice-cold lysis buffer, the immunoprecipitated proteins were eluted from the beads with sodium dodecyl sulfate (SDS) sample loading buffer. The eluted samples were then separated by SDS–polyacrylamide gel electrophoresis (PAGE) gels and visualized via Western blot analysis. HRP conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham Biosciences (NJ, USA). The polyclonal anti-HA antibody (1:1000) and the polyclonal anti-His (1:1000) were from MBL (Medical and biological laboratories, Japan). The polyclonal anti-mGluR1a (1:500) was purchased from Chemicon (Temecula, CA, USA). The anti-CAL antibody (1:1000) was from ProSci incorporated. The polyclonal anti-ERK (1:2000) and monoclonal anti-pERK (1:2000) were purchased from Upstate Biotechnology (NY, USA).

2.5. GST fusion protein pull-down assay

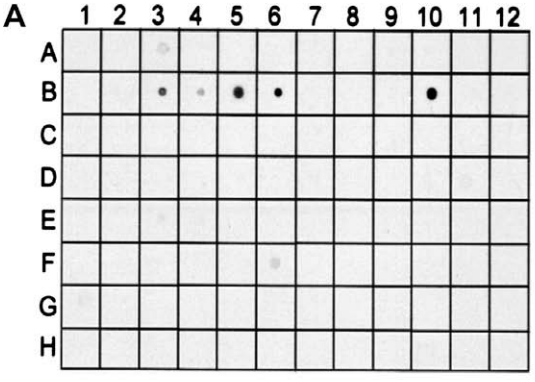
GST fusion proteins were purified from bacteria using Glutathione Sepharose 4B beads (Sigma–Aldrich, UT, USA) according to the manufacturer's protocol. Briefly, equal amounts of GST and GST-mGluR1a-CT fusion proteins (conjugated on beads) were incubated with an equal concentration of His-CAL fusion protein from bacteria or cell lysates from COS-7 cells over-expressing HA-CAL. The beads were washed with wash buffer I (10 mM HEPES, 100 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.1% Tween 20, 3% BSA, pH 7.4) and wash buffer II (10 mM HEPES, 100 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.1% Tween 20, pH 7.4). The proteins were eluted with 1 × SDS–PAGE sample buffer and resolved via SDS–PAGE, and the pulled-down CAL was detected by Western blot.

2.6. Immunofluorescence microscopy

The method for immunofluorescence has been described previously [14]. Briefly, cells were fixed, permeabilized, and incubated with anti-Flag and anti-HA antibodies, followed by incubation with a rhodamine-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG. Nuclei were stained with Hoechst 33258 and visualized by a confocal microscope (Leica Microsystems, LAS AF-TCS SP5).

2.7. Phospho-ERK assay

The assay for ERK activation has been described previously [14]. Briefly, cells were transfected and incubated in serum-free media overnight prior to experiments. Following a 5-min agonist stimulation, cells



B

Bin	PDZ Domain	Bin	PDZ Domain	Bin	PDZ Domain
A1	MAGI-1 PDZ1	C9	ERBIN PDZ	F5	MUPP1 PDZ10
A2	MAGI-1 PDZ2	C10	ZO-1 PDZ1	F6	MUPP1 PDZ12
A3	MAGI-1 PDZ3	C11	ZO-1 PDZ2	F7	MUPP1 PDZ13
A4	MAGI-1 PDZ4+5	C12	ZO-1 PDZ3	F8	PTPN13 PDZ1
A5	MAGI-2 PDZ1	D1	ZO-2 PDZ1	F9	PTPN13 PDZ3
A6	MAGI-2 PDZ2	D2	ZO-2 PDZ2	F10	PTPN13 PDZ4+5
A7	MAGI-2 PDZ3	D3	ZO-2 PDZ3	F11	PDZK1 PDZ1
A8	MAGI-2 PDZ4	D4	ZO-3 PDZ1	F12	PDZK1 PDZ2
A9	MAGI-2 PDZ5	D5	ZO-3 PDZ2	G1	PDZK1 PDZ3
A10	MAGI-3 PDZ1	D6	ZO-3 PDZ3	G2	PDZK1 PDZ4
A11	MAGI-3 PDZ2	D7	C2PA PDZ	G3	PDZK2 PDZ1
A12	MAGI-3 PDZ3	D8	GIPC PDZ	G4	PDZK2 PDZ2
B1	MAGI-3 PDZ4	D9	MALS-1 PDZ	G5	PDZK2 PDZ3
B2	MAGI-3 PDZ5	D10	MALS-3 PDZ	G6	PDZK2 PDZ4
B3	NHERF-1 PDZ1	D11	Densin-180 PDZ	G7	LNX1 PDZ1
B4	NHERF-1 PDZ2	D12	Rhophilin-1 PDZ	G8	LNX1 PDZ2
B5	NHERF-2 PDZ1	E1	Rhophilin-2 PDZ	G9	LNX1 PDZ3
B6	NHERF-2 PDZ2	E2	Harmonin PDZ1	G10	LNX1 PDZ4
B7	PSD-95 PDZ1+2	E3	Harmonin PDZ2	G11	LNX2 PDZ1
B8	PSD-95 PDZ3	E4	Neurabin PDZ	G12	LNX2 PDZ2
B9	CNRasGEF PDZ	E5	Spinophilin PDZ	H1	LNX2 PDZ4
B10	CAL PDZ	E6	α1-syntrophin PDZ	H2	PTPN4 PDZ
B11	nNOS PDZ	E7	ß1-syntrophin PDZ	H3	Rho-GEF PDZ
B12	INADL PDZ5	E8	ß2-syntrophin PDZ	H4	RA-GEF PDZ
C1	INADL PDZ6	E9	γ1-syntrophin PDZ	H5	Enigma PDZ
C2	SAP97 PDZ1+2	E10	γ2-syntrophin PDZ	H6	LARG PDZ
C3	SAP97 PDZ3	E11	PAPIN PDZ	H7	PTPN3 PDZ
C4	SAP102 PDZ1+2	E12	MAST-205 PDZ	H8	Shank1 PDZ
C5	SAP102 PDZ3	F1	MUPP1 PDZ1	H9	Tamalin PDZ
C6	Chap110 PDZ1+2	F2	MUPP1 PDZ6	H10	PAR-3 PDZ1
C7	Chap110 PDZ3	F3	MUPP1 PDZ7	H11	PAR-3 PDZ2
C8	E6TP1 PDZ	F4	MUPP1 PDZ8	H12	PAR-3 PDZ3

Fig. 1. Identification of mGluR1a-CT binding partners by PDZ array. (A) A proteomic array containing 96 putative class 1 PDZ domains was overlaid with 100 nM GST fusion proteins corresponding to the last 25 amino acids of mGluR1a. (B) 96 putative class 1 PDZ domains corresponding to the proteomic array.

were harvested in SDS sample loading buffer. The levels of p42/44 ERK phosphorylation and total ERK were visualized by Western blot using an anti-phospho-ERK1/2 antibody and anti-ERK antibody, respectively (Upstate Biotechnology, USA). Immunoreactive bands were visualized via chemiluminescence and quantified using NIH Image 1.62. For each sample, the level of phospho-ERK immunoreactivity was normalized to total ERK immunoreactivity.

3. Results

3.1. CAL is identified as a novel binding partner of mGluR1a in vitro

To identify PDZ domain-containing proteins that might associate with mGluR1a, GST-mGluR1a-CT, which possesses a PDZ-interaction motif, was prepared and subsequently used in overlay experiments. As shown in Fig. 1, mGluR1a-CT interacted with several of the PDZ domains on the array, including NHERF-1 PDZ1+2 (B3, B4), and NHERF-2 PDZ1+2 (B5, B6), which have previously been reported [15]. The overlays of the PDZ array also revealed the robust interaction of mGluR1a-CT with CAL (B10) (Fig. 1A). In contrast to GST-mGluR1a-CT, there was no detectable binding of GST alone, demonstrating the feasibility of the analytical assay for the screening of proteomic arrays (data not shown).

To test the direct association of mGluR1a-CT with CAL, we employed a fusion protein pull-down approach. As expected, GST-mGluR1a-CT was found to robustly pull-down the PDZ domain of CAL from bacterial lysates (Fig. 2A). Furthermore, HA-tagged full-length CAL from the transiently transfected COS-7 cells was also shown to associate with mGluR1a-CT (Fig. 2B). We further explored the interaction of endogenous CAL with mGluR1a-CT in rabbit brain (Fig. 2C), and the results were consistent with the observation in the cell lysates. Mutation of the terminal Leu residue to Ala on mGluR1a completely blocked the binding to CAL, demonstrating that the PDZ-domain binding motif in the C-terminal of mGluR1a is required for the interaction (Fig. 2B).

To assess the affinity of the mGluR1a-CAL interaction, saturation-binding studies were performed (Fig. 2D). The binding affinity of CAL-PDZ and mGluR1a-CT was estimated at 75 nM, revealing that this interaction is of relatively high affinity. Taken together, these results demonstrated the specific interaction of mGluR1a-CT with the PDZ domain of CAL in vitro.

3.2. mGluR1a associates with CAL in cells and native tissues

To determine whether the interaction between full-length mGluR1a and full-length CAL occurs in a cellular context, we transfected HA-CAL with either Flag-mGluR1a-WT or Flag-mGluR1a-deleted carboxyl terminus (ΔCT) in COS-7 cells, respectively. Immunoprecipitation of mGluR1a followed by Western blotting for CAL revealed that CAL existed in mGluR1a-WT immunoprecipitation complex (Fig. 3A), and that the C terminus deleted mutation sharply reduced the ability of the receptor to co-immunoprecipitate CAL. These results indicate that full-length mGluR1a and CAL interact in cells and that the C terminus of the mGluR1a is a crucial determinant of the CAL-mGluR1a association. We next tested the agonist-regulated interaction between mGluR1a and CAL. As shown in Fig. 3B, following 30-min stimulation with a

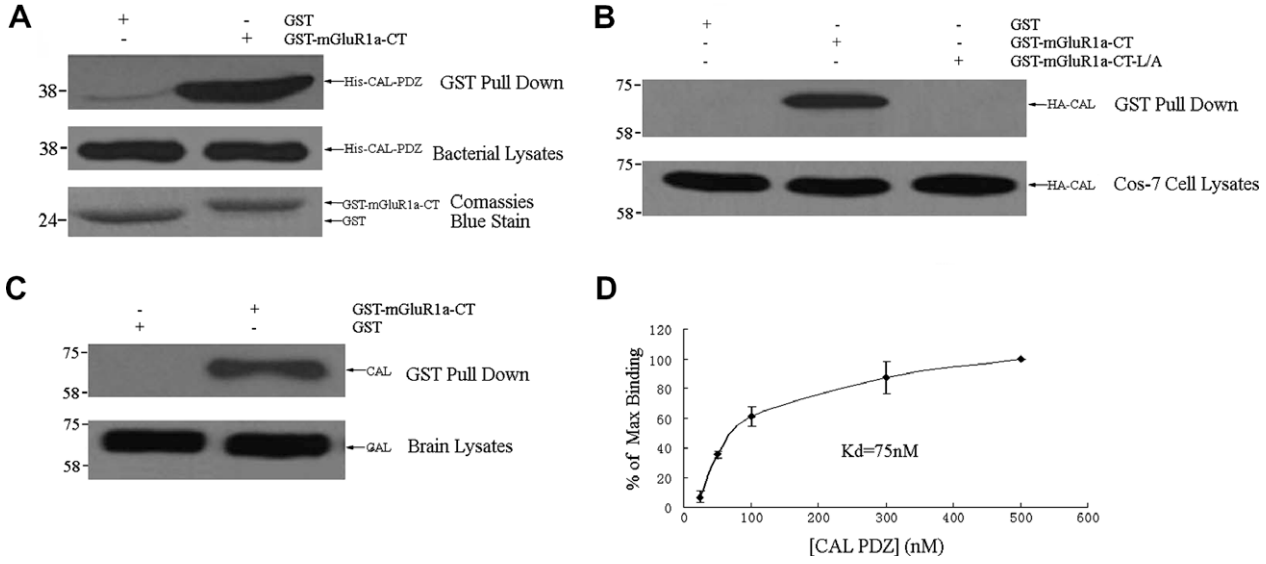


Fig. 2. mGluR1a-CT binds specifically and with high affinity to the PDZ domain of CAL. (A) mGluR1a-CT binds with the PDZ domain of CAL in bacterial induction. GST or GST fusion proteins corresponding to mGluR1a-CT were adsorbed to glutathione–agarose beads and used to pull-down His-CAL-PDZ fusion protein from bacterial lysates. The pulled-down CAL was detected with anti-His antibody via Western blot (top). The same amount of His-CAL-PDZ fusion protein was used (middle), and Coomassie blue staining revealed the equal loading of GST and GST fusion proteins (bottom). (B) mGluR1a-CT associates with full-length HA-CAL in transiently transfected COS-7 cells. The pulled-down complex from GST or GST fusion proteins corresponding to mGluR1a-CT and mGluR1a-CT-L/A (mutant) were visualized with anti-HA antibody. (C) mGluR1a-CT interacts with endogenous CAL in rabbit brain. 'The pulled-down complexes from GST-mGluR1a-CT or GST alone fusion proteins were visualized with anti-CAL antibody. (D) The overlay assay revealed that the interaction between mGluR1a-CT and CAL-PDZ is of high affinity. Nitrocellulose strips containing 2 μg of His-CAL-PDZ were incubated with GST-mGluR1a-CT at five concentrations between 1 and 500 nM. Specific binding of mGluR1a-CT did not increase between 300 and 500 nM, and thus the binding observed at 500 nM was defined as "maximal" binding. The binding observed at the other concentrations was expressed as a percentage of maximal binding within each experiment. The bars indicate mean ± S.E. from four independent experiments.

selective mGluRI agonist DHPG (100 μM), the amount of co-immunoprecipitated CAL was approximately six times that co-immunoprecipitated under basal conditions, revealing that the interaction can be promoted (or perhaps stabilized) by agonist activation of the mGluR1a.

In addition to transfection experiments, we also studied the interactions of endogenous mGluR1a and CAL in native tissue via the reverse Co-IP (Fig. 3C). Solubilized lysates derived from rat brain tissues were incubated with anti-CAL antibody to immunoprecipitate CAL protein, and the resultant immunoprecipitates were probed for mGluR1a using an anti-mGluR1a antibody. In agreement with the transfected cell experiments, robust co-immunoprecipitation of mGluR1a with CAL was observed in brain tissues. These data reveal the existence of a physical complex between CAL and mGluR1a in native tissues, in which neither protein is over-expressed.

Further evidence for a cellular mGluR1a/CAL complex came from double-immunofluorescence studies, revealing the colocalization of Flag-mGluR1a with HA-CAL in cells. BHK cells were transfected with mGluR1a in the absence or presence of CAL, and the immunostaining of both proteins was analyzed. Under the basal level, we observed a

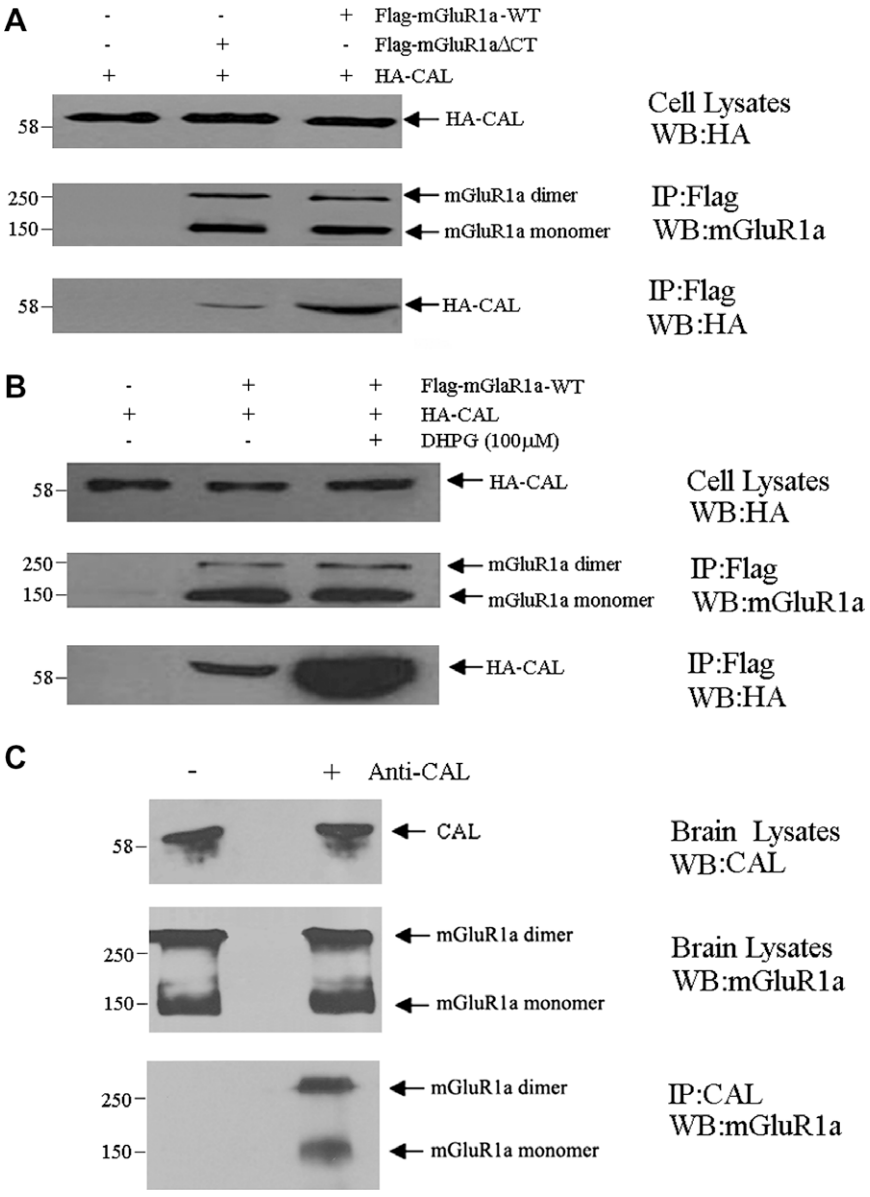


Fig. 3. Co-immunoprecipitation of CAL with Flag-tagged full-length mGluR1a is enhanced by agonist stimulation. (A) Cellular association of full-length Flag-mGluR1a with HA-CAL. Lysates from transfected COS-7 cells with the corresponding plasmids were incubated with anti-Flag antibody coupled to beads to immunoprecipitate Flag-tagged receptors (middle), and the precipitated complexes were probed with anti-HA antibody via Western blot to detect the interaction of CAL (bottom). Lysates were probed with anti-HA antibody to visualize the equal expression of CAL (top). (B) The association of mGluR1a and CAL was promoted by stimulation with DHPG. Before harvesting, transfected cells were incubated in the absence or presence of DHPG (100 μM, 30 min). (C) mGluR1a and CAL interact in native tissues. Solubilized lysates from homogenized rat brain tissue were subjected to immunoprecipitation with anti-CAL antibody. The co-immunoprecipitated mGluR1a was then probed with anti-mGluR1a antibody via Western blot (bottom). Lysates were probed with anti-CAL antibody (top) and anti-mGluR1a antibody (middle) to visualize the equal amount of CAL and receptors, respectively. IP, immunoprecipitation; WB, Western blot. Data are representative of three independent experiments.

"thick" rim distributed by mGluR1a around the cell either in the absence or presence of CAL, as assessed on the cells permeabilized with saponin (Fig. 4A and D), and CAL was observed predominantly as multiple spots in the cytoplasm (Fig. 4E), overlapping with the expression of the golgi-associated protein GM 130 (data not shown). Unexpectedly, no

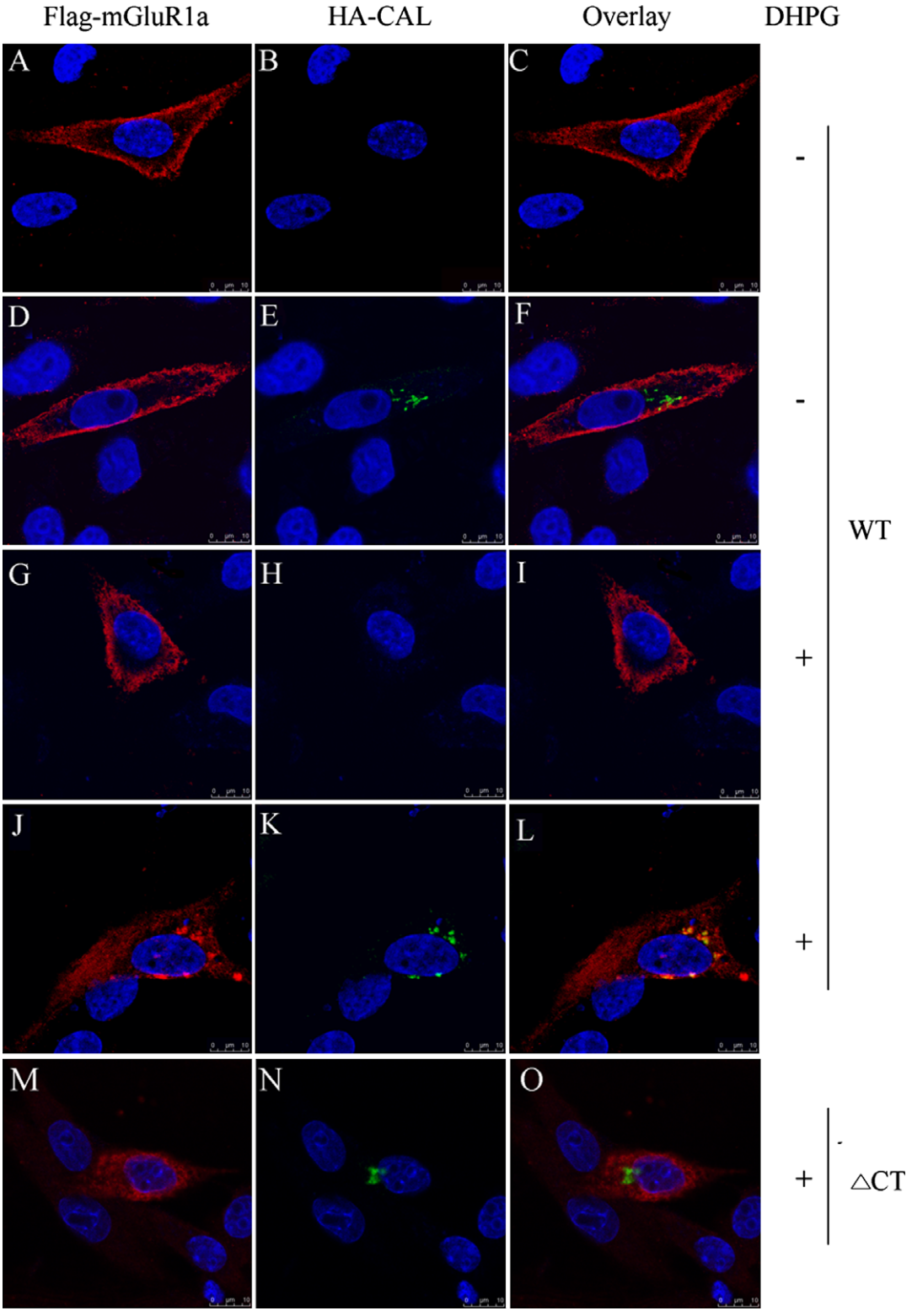


Fig. 4. CAL was co-localized with mGluR1a in the immunofluorescence assay. BHK cells were transiently transfected with Flag-mGluR1a-WT alone (A–C, G–I) or Flag-mGluR1a-WT/HA-CAL (D–F, J–L) in the presence or absence of DHPG (100 μM), or Flag-mGluR1a-ΔCT/ HA-CAL (M–O) with DHPG (100 μM). Colocalization of Flag-mGluR1a-WT with HA-CAL is shown in yellow in the overlaid image (L). Data are representative of three independent experiments.

striking overlap of mGluR1a and CAL was detected under the basal level (Fig. 4F). Following stimulation with DHPG (100 μM), the ''thicker'' rim distributed by mGluR1a around the cells was formed, indicating that mGluR1a alone was partially re-distributed in the cytoplasm, consistent with the receptor internalization (Fig. 4G and I). In cells co-transfected with CAL, CAL remained in the cytoplasm as multiple spots (Fig. 4K) and multiple spots of mGluR1a immunostaining were also found in the cytoplasm (Fig. 4J), where it was co-localized with CAL (Fig. 4L). The apparent expression levels of mGluR1a and CAL were unchanged after stimulation with DHPG detected by Western blot (data not shown). Conversely, when Flag-mGluR1a-ΔCT was co-expressed with CAL followed by DHPG treatment, the mutant receptor did not exhibit the overlap with CAL immunolabeling in the cytoplasm (Fig. 4M–O). The distribution of the mutant receptor transfected alone was similar to that of the wild-type receptor (data not shown). There was no significant colocalization between mutant mGluR1a and CAL. In the immunofluorescence experiment, the interaction of mGluR1a with CAL was promoted by DHPG stimulation, consistent with our co-immunoprecipitation data, suggesting that CAL regulated the intracellular trafficking of mGluR1a.

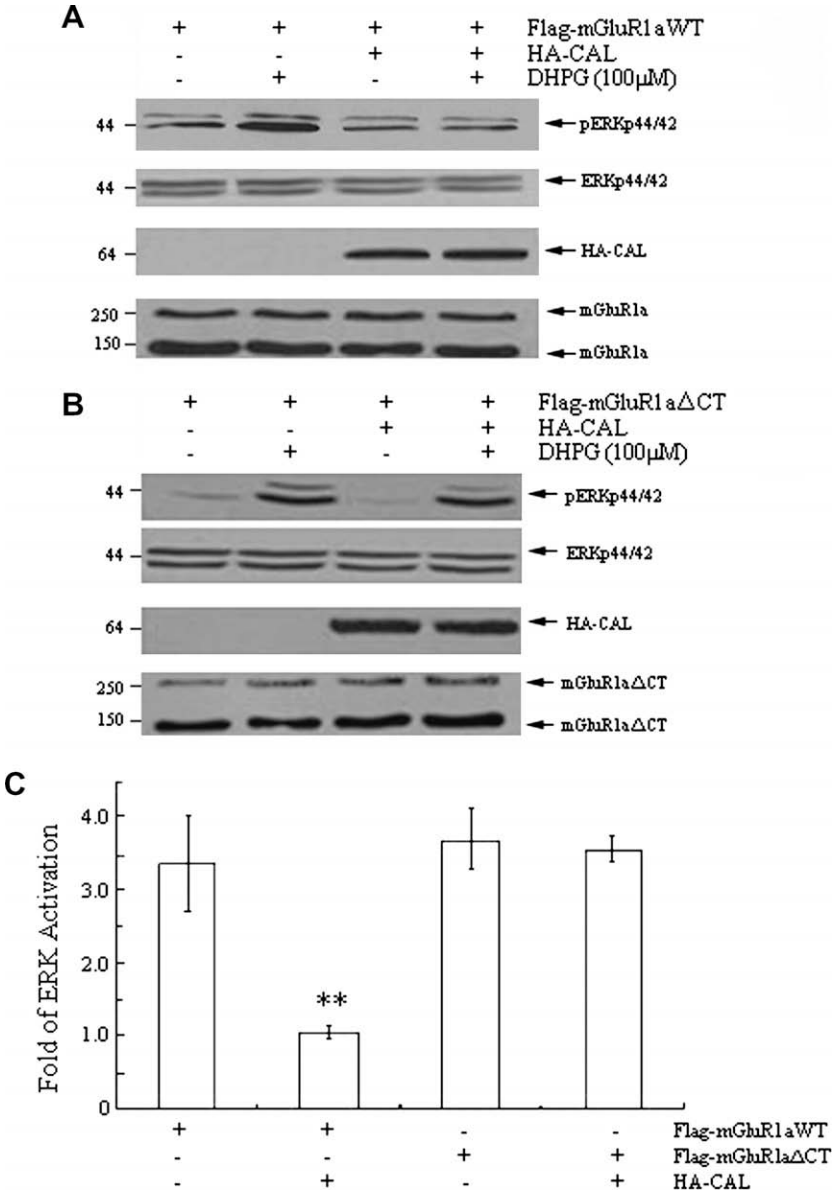


Fig. 5. CAL attenuated mGluR1a-mediated ERK activation. (A) CAL inhibited wild-type mGluR1a-mediated ERK activation. COS-7 cells were transiently transfected with Flag-mGluR1a-WT in the absence or presence of HA-CAL. Twenty-four hours after transfection, cells were treated with serum-free medium overnight. After the cells were stimulated with DHPG (100 μM, for 5 min at 37 °C), they were harvested and analyzed for ERK activity by Western blot. (B) CAL had no apparent effect on mGluR1aΔCT-mediated ERK activation. COS-7 cells were transiently transfected with Flag-mGluR1aΔCT alone or Flag-mGluR1aΔCT/HA-CAL, and analyzed for the ERK activity. (C) Quantification of the effect of CAL on Flag-mGluR1a-WT and Flag-mGluR1a-ΔCT-mediated ERK activation. The amount of phospho-ERK immunoreactivity observed following DHPG stimulation was expressed as the fold difference relative to phospho-ERK immunoreactivity in the absence of stimulation. The data represent means ± S.E. from four independent experiments. The asterisks indicate a significant decrease ($P < 0.01$) relative to phospho-ERK immunoreactivity in the absence of transfected CAL.

3.3. CAL modulates mGluR1a-mediated ERK activation

Activation of mGluR1a can stimulate ERK phosphorylation [16,17]. To determine the functional linkage with the interaction, we investigated the effect of CAL expression on the mGluR1a-mediated ERK activation. As shown in Fig. 5, DHPG-stimulated ERK activation was more than three fold with that mGluR1a expressed alone, but it was almost blocked when mGluR1a was co-expressed with the CAL protein (Fig. 5A, 5C). In contrast, CAL had no significant effect on the ability of mGluR1aΔCT to couple to ERK activation (Fig. 5B). The total expression levels of mGluR1a and CAL remained unchanged after agonist stimulation (data not shown).

4. Discussion

CAL is known to be predominantly located in the golgi apparatus, to interact with syntaxin 6, a Q-SNARE protein that is involved in trafficking between endosomes and the *trans*-golgi network [18], and to assist in the proper sorting of membrane proteins [11,19]. Thus, CAL might be a general sorting molecule that assists membrane–receptor trafficking. DHPG robustly stimulates the association of mGluR1a with CAL (Fig. 3), and it promotes the colocalization of mGluR1a and CAL in the cytoplasm (Fig. 4L). The receptor activation with DHPG triggers mGluR1a endocytosis, and consequently, more receptors could be present in CAL-containing compartments, in which the interaction of CAL may retard mGluR1a recycling back to the plasma membrane. This could result in a block of the receptor signaling cascade, such as ERK activation observed here (Fig. 5), but the exact mechanism requires further investigation. Indeed, there are observations to support the hypothesis. For example, CAL overexpression has been shown to retard the β1-adrenergic receptor [20], and the co-expression of the mGluR1a mutant receptor did not colocalize with CAL in the cytoplasm (Fig. 4). In non-stimulated conditions, we observed both "thick" rims distributed by mGluR1a around the cells either in the absence or presence of CAL, as assessed on the cells permeabilized with saponin (Fig. 4A and D). This might be due to the constitutive internalization of mGluR1a [21], causing the effect of CAL on the mGluR1a distribution to become invisible under the condition. Indeed, a "thin" rim of nonpermeabilized cell-surface mGluR1a was observed by immnostained with antibody against the extracellular domain of mGluR1a [8]. Meanwhile, we did not detect a significant colocalization under non-stimulated conditions. Our double-immunofluorescence assay might not have been sensitive enough to detect a small population of co-localized mGluR1a and CAL under the conditions used here.

Compared with its shorter C-terminal variants, mGluR1a displays many different functional properties [22,23]. Noticeably, of the mGluR1 subfamily, mGluR1a possesses the longest C terminus of the six different isoforms of mGluR1 (mGluR1a-1f), which is the distinct structure containing the PDZ-binding motif that binds to the PDZ proteins. Indeed, mGluR5, highly homologous to mGluR1a, is also found to interact with scaffold proteins [20], including CAL (our unpublished data). It has been reported that mGluR1a associates with the PDZ protein tamalin to cause an increase in cell-surface expression of mGluR1a in COS-7 cells [8]. Here, we found that CAL interaction with mGluR1a, but not with other isoforms deficient of the PDZ-binding motif such as mGluR1b (data not shown), inhibited the ERK activation mediated by mGluR1a. The PDZ-binding proteins with mGluR1a may help to explain the differences in mGluR1a behaviors.

In summary, mGluR1a via its C terminus interacts with the PDZ domain of CAL. The association of mGluR1a with CAL functionally inhibits the mGluR1a-mediated ERK activation. Since mGluR1a and CAL are abundantly expressed in the brain, in the future study, it would be interesting to explore how CAL interaction regulates mGluR1a activity in the neuron system, and to further explore how mGluR1a association with CAL functionally cooperates with other mGluR1a interacting proteins. Our findings add a novel binding partner to the mGluRI interacting protein family, thus opening a window of understanding to the receptor activity regulation through the interaction with CAL.

Acknowlegements: We thank Randy Hall and Maryse Paquet (Emory University, GA, USA) for technical assistance and advices in the early stages of this project.

This work was supported by the National Natural Science Foundation of China (Nos: 30572170, 30572183, 30873087 and 30772573) and the Natural Science Foundation of Beijing (Nos: 7082010 and 7082009), the New Century Excellent Talents in University of China (NCET-06-0184), the Research Fund for the Doctoral Program of Higher Education of China (20060025011), the Foundation of Beijing Educational Committee (KZ200610025013), the Funding Project for Academic Human Resources Development in Institutions of Higher Learning under Jurisdiction of Beijing Municipality PHR (IHLB), the Scientific Research Foundation for Returned Scholars, the Ministry of Education of China, as well as the Beijing Science and Technique New Star (2007B065).

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